Chapter 8

Agrobacterium tumefaciens-Mediated Transient Transformation of *Arabidopsis thaliana* Leaves

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Abstract

Transient assays provide a convenient alternative to stable transformation. Compared to the generation of stably transformed plants, agroinfiltration is more rapid, and samples can be analyzed a few days after inoculation. Nevertheless, at difference of tobacco and other plant species, *Arabidopsis thaliana* remains recalcitrant to routine transient assays. In this chapter, we describe a transient expression assay using simple infiltration of intact *Arabidopsis* leaves with *Agrobacterium tumefaciens* carrying a plasmid expressing a reporter fluorescent protein. In this protocol, *Agrobacterium* aggressiveness was increased by a prolonged treatment in an induction medium deficient in nutrients and containing acetosyringone. Besides, *Arabidopsis* plants were cultivated in intermediate photoperiod (12 h light–12 h dark) to promote leaf growth.

Key words Transient gene expression, *Arabidopsis thaliana*, *Agrobacterium tumefaciens*, Leaf agroinfiltration, Fluorescent proteins

1 Introduction

Stable transgenic *Arabidopsis* offer advantages in terms of a sustainable supply of plant material with homologous protein expression, the potential of mutant complementation, as well as a global examination option throughout all tissues and cell types. Although the often used floral dip procedure [1] generates transgenic *Arabidopsis* plants with minimal labor, plants must still be grown to maturity over several weeks. The need to harvest seed and perform selection also makes it impractical to test large numbers of different transgene constructs. Moreover, transgene expression in some cases could interfere with normal plant growth and development due to an overdose of the functional proteins or dominant negative effect of nonfunctional products. Transient gene expression provides a convenient alternative to stable transformation in analyzing gene function by virtue of its time and labor efficiency.

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It only takes one to several days to perform the assay in its entirety, which allows many constructs to be assayed in parallel within a short time and dramatically speeds up the pace of research.

Transient infiltration assays with *Agrobacterium* carrying a construct of interest are a powerful tool to gain inside into gene function, protein-protein interaction analysis, and promoter analysis [2–4]. *Agrobacterium*-mediated transient transformation is an easy, routine, and consistent operation in *Nicotiana benthamiana* leaves [2], and the procedure has also been adjusted to lettuce and tomato leaves [3, 5], as well as tomato fruits [6], roots [7], Antirrhinum floral tissues [8], and whole seedlings [9]. At difference of tobacco and other plant species, *Arabidopsis* still remains recalcitrant to routine transient assays, and high transient expression levels are obtained only in some ecotypes [3, 9–12]. However, when used as a heterologous system to express genes from the model species *Arabidopsis*, tobacco may not reflect the native activity or subcellular distribution of the corresponding proteins [10]. Pilot efforts to explore an *Arabidopsis* equivalent of tobacco leaf infiltration have demonstrated low-frequency success with great variation [3, 4, 13, 14].

Efforts to increase the frequency of *Arabidopsis* transient transformation success and also to decrease variation using young seedlings [10], as well as transient transformation of root epidermal cells by cocultivation with *Agrobacterium rhizogenes* [15], have been described. Difficulties in *Arabidopsis* transient transformation have been attributed to plant immune responses triggered by perception of Agrobacterium [12]. Using transgenic *Arabidopsis* expressing AvrPto (a suppressor of plant immunity from *Pseudomonas syringae*) under the control of a dexamethasone inducible promoter, an efficient *Agrobacterium*-mediated transient transformation method of *Arabidopsis* has been developed [12]. Nevertheless, this assay is limited to the use of transgenic plants expressing AvrPto.

In this chapter, we describe a transient expression assay using simple infiltration of intact *Arabidopsis* leaves with *Agrobacterium tumefaciens GV3101* cells carrying appropriate plasmid constructs. This protocol increases *Agrobacterium* aggressiveness by a prolonged treatment in the presence of acetosyringone (AS) and medium deficient in nutrients such as the induction one. In addition, the number of bacteria used is higher than the one used to infiltrate *Nicotiana benthamiana* leaves. Finally, *Arabidopsis* growing conditions are controlled in order to obtain healthy plants with an adequate leaf size to facilitate infiltration. We showed that a fluorescent reporter gene is easily introduced in *Arabidopsis* leaves and that most of the epidermal cells show fluorescence when fluorescence microscope and Confocal Laser Scanning Microscopy (CLSM) are used.

2 Materials

1. Seeds of *Arabidopsis thaliana* Columbia (Arabidopsis Stock Center).
2. Pots (≈6 cm of diameter), compost, and perlite.
3. *Agrobacterium tumefaciens GV3101* (strain that contains the sequences derivative of the nopaline-type disarmed Ti-plasmid pTiC58 and rifampicin resistance gene integrated on the chromosome and the helper plasmid pMP90 (pTiC58ΔT-DNA) with a gentamicin resistance gene) [16].
4. Binary vector carrying the gene of interest (Gi) (e.g., cloned into pGWB, a group of vectors designed to facilitate fusions to different reporter proteins and also purification and detection tags [17]).
5. Kanamycin (Sigma-Aldrich) 1,000× stock solution: 100 mg/mL in water.
6. Gentamicin (Sigma-Aldrich) 1,000× stock solution: 30 mg/mL in water.
7. Rifampicin (Sigma-Aldrich) 1,000× stock solution: 10 mg/mL in methanol.
8. Bacterial culture medium: YEB (yeast extract and beef) medium (Sigma-Aldrich). Add 18 g/l agar–agar for solid medium.
9. Glycerol solutions: 10 % and 80 % v/v in water.
10. Induction medium: 0.1 % $(NH4)_2SO_4$, 0.45 % KH_2PO_4, 1 % K_2HPO_4, 0.05 % sodium citrate, 0.2 % sucrose, 0.5 % glycerol, 1 mM $MgSO_4$, and pH 5.7.
11. Infiltration medium: MES (Sigma-Aldrich) 10 mM, $MgSO_4$ 10 mM, and pH 5.7.
12. Acetosyringone: (Sigma-Aldrich): 200 mM in dimethyl sulfoxide (DMSO).
13. Perfluorodecalin 95 % (Sigma-Aldrich).
14. Syringes 1 mL.
15. Shaker.
16. Spectrophotometer.
17. Refrigerated centrifuge.
18. Gene Pulser II with the Capacitance Extender (Bio-Rad).
19. Microcentrifuge.
20. Fluorescence stereomicroscope equipped with a GFP Plant (excitation 470/40 nm, emission 525/50 nm) and DsRed (excitation 545/30 nm, emission 620/60 nm) filters and CCD camera.
21. Confocal laser scanning microscope with a 63× (NA 1.4) oil immersion objective.

3 Methods

3.1 Growing Arabidopsis Plants

1. Fill the 6 cm pots with a mix of compost and perlite (3:1), and compress very lightly to give a firm bed and water.
2. Sow the seeds onto the surface of the mix compost/perlite by scattering them carefully.
3. Place the pots in a tray and transfer to a cold (4 °C) for 2–3 days in the dark, and cover with transparent PVC film to keep them in a high humidity environment.
4. Transfer the pots to a growth room under 90 μE in light cycle 12 h light–12 h dark at 22–24 °C (*see* **Note 1**).
5. After 4 weeks, *Arabidopsis* plants are generally in good conditions for transient expression assay (Fig. 1a) (*see* **Note 2**).

3.2 Transformation of A. tumefaciens with Binary Plasmid DNA by Electroporation

3.2.1 Preparation of Competent Cells of Agrobacterium

1. Pick a single colony of the *A. tumefaciens* GV3101 and inoculate 3 mL of YEB with gentamicin 30 μg/mL and rifampicin 10 μg/mL in a 15 mL sterile tube. Grow at 28 °C overnight in a shaker at 200 rpm in the dark.
2. Inoculate 500 mL flasks each containing 100 mL of YEB with 0.5 mL (1/100 volume) of the overnight culture and grow at 28 °C with vigorous shaking until OD_{600nm} of 0.5–0.6. It takes ~4–5 h to get the cells to this stage.
3. Spin 5 min at 5,000 × *g* at 4 °C. Pour off supernatant.
4. Resuspend cells in 50 mL (~1/2 volume) ice-cold 10 % glycerol. Repeat spin.
5. Resuspend cells in 25 mL of ice-cold 10 % glycerol. Repeat spin.
6. Resuspend cells in 12 mL of ice-cold 10 % glycerol. Repeat spin.
7. Resuspend final pellet in 1.5 mL ice-cold 10 % glycerol.

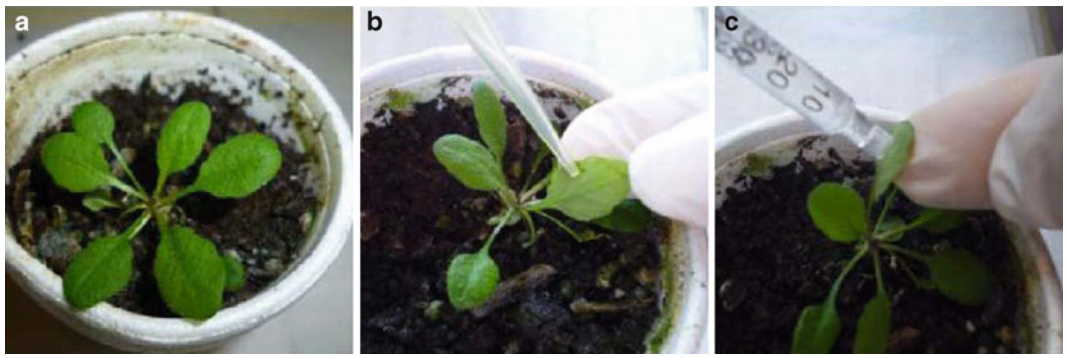

Fig. 1 (**a**) *Arabidopsis* 4-week-old plants. (**b**) Using a *yellow* tip, create small holes in the leaves. (**c**) Press the nozzle of a 1 mL syringe against the lower (abaxial) epidermis of *Arabidopsis* leaf

8. Dispense 100 µL aliquots into fifteen 1.5 mL microfuge tubes pre-chilled on ice. Each tube will have enough cells for 2 transformations.
9. Quick-freeze the tubes in liquid nitrogen and store at −80 °C.

3.2.2 Electroporation

1. Remove one tube of competent cells from the freezer and place it on ice. Allow to thaw slowly on ice.
2. Add 1–2 µL of DNA (50–100 ng in water) and wait for 1 min.
3. Transfer cells plus DNA to pre-chilled (on ice) electroporation cuvettes with either 1 or 2 mm gap sizes. Make sure the white cuvette holder from the Bio-Rad Gene Pulser II is also pre-chilled on ice.
4. Take the ice bucket with the cuvettes and cuvette holder to the Gene Pulser. For cuvettes with a 2 mm gap size, adjust the Gene Pulser II unit "Set Volts" setting to 2.5 kV and the capacitance setting to 25 µFD. Set the resistance to 200 Ω on the Pulse Controller Unit.
5. Place the cuvette in the cuvette holder, slide down to engage the electrodes, and push both buttons on the Gene Pulser, holding them until the tone sounds.
6. Add 500 µL of YEB medium directly to the cuvette immediately after the pulse and incubate in a shaker at 200 rpm and 28 °C overnight.
7. Plate 100–200 µL on selective media (i.e., antibiotic selection for both the bacterial host strain and the plasmid).
8. Incubate plates 2 days at 28 °C when the colonies should be visible.
9. Check the presence of the introduced vector by a Colony PCR (*see* **Note 3**).
10. Grow a single colony in 5 mL YEB with gentamicin (30 µg/mL), rifampicin (10 µg/mL), and kanamycin (100 µg/mL) in the dark at 28 °C and 200 rpm (*see* **Note 4**).
11. Store as glycerol stock (800 µL of fresh overnight culture + 200 µL sterile 80 % glycerol) at −80 °C (*see* **Note 5**).

3.3 Agrobacterium Growing for Infiltration

1. Plate 100–200 µL of a glycerol stock on YEB medium with 30 µg/mL gentamicin, 10 µg/mL rifampicin, and 100 µg/mL kanamycin (if the Gi is in a kanamycin resistance binary vector such as pGWB [17]). After incubation at 28 °C, pick a single colony of the *Agrobacterium tumefaciens* GV3101 containing the plasmid of interest and inoculate 5 mL of YEB with antibiotics. Grow at 28 °C overnight in a shaker at 200 rpm in the dark.
2. Dilute the overnight culture in YEB with antibiotics to reach an absorbance OD_{600nm} of approximately 0.3 and add acetosyringone at 100 µM for virulence gene induction. Incubate at 28 °C and 200 rpm until the culture reach OD_{600nm} of 0.6.

3. Spin the culture at 5,000 × *g* for 5 min.
4. Resuspend in 5 mL induction medium supplemented with antibiotics and acetosyringone at 200 μM. Incubate at 30 °C and 200 rpm for 3–4 h.
5. Pellet the culture at 5,000 × *g* for 5 min in a microcentrifuge at room temperature.
6. Resuspend the pellet in 5 mL of infiltration medium and centrifuge as above. Repeat once.
7. Dilute the bacterial suspension with infiltration medium supplemented with acetosyringone at 200 μM to adjust the inoculum to an appropriate concentration (*see* **Note 6**).

3.4 Transient Gene Expression

1. Agroinfiltration is conducted by infiltrating the agrobacterial suspension into the abaxial surface of fingernail-sized leaves attached to the intact plant (*see* **Note 7**). Using a yellow tip, make small holes in the leaves (Fig. 1b).
2. Load the inoculum in 1 mL plastic syringe and press the nozzle of the syringe (no needle) against the lower (abaxial) epidermis of an *Arabidopsis* leaf, covering the small hole with the nozzle and holding the leaf with a gloved finger on the adaxial face. Introduce the *Agrobacterium* in infiltration medium by slowly injection (Fig. 1c) (*see* **Note 8**).
3. Using a glass permanent maker, mark the infiltrated region.
4. Place the infiltrated *Arabidopsis* plants in the growth room (light cycle 12 h light–12 h dark at 22–24 °C) for 2–5 days.
5. If the plants were infiltrated with *Agrobacterium* with a fluorescent reporter, check the presence of the fluorescent protein (FP) using fluorescence stereomicroscope equipped with an appropriated filters (Fig. 2). Exposition time should be adjusted with a no transformed leaf (Fig. 2a) to distinguish the FP signal from the autofluorescent (Fig. 2b) (*see* **Note 9**).

3.5 Confocal Imaging

1. Excise a marked area of the leaf and mount it on a glass microscope slide containing a few drops of water.
2. Fill a 1 mL plastic syringe with a needle with perfluorodecalin, drop it over the leaf, and place the cover glass over the leaf (*see* **Note 10**).
3. Examine with a confocal laser scanning microscope, using a 63× (NA 1.4) oil immersion objective (*see* **Note 11**). GFP was excited at 488 nm (Ar 100 mW Laser) and detected in the 496–532 nm range. YFP was excited at 514 nm (Ar 100 mW Laser) and detected in the 525–559 nm range (Fig. 3a). mCherry and RFP were excited at 543 nm (HeNe 1.5 mW laser) and detected in the 570–630 nm range (Fig. 3b). To analyze colocalization, combine both channels (Fig. 3c) (*see* **Notes 12** and **13**).

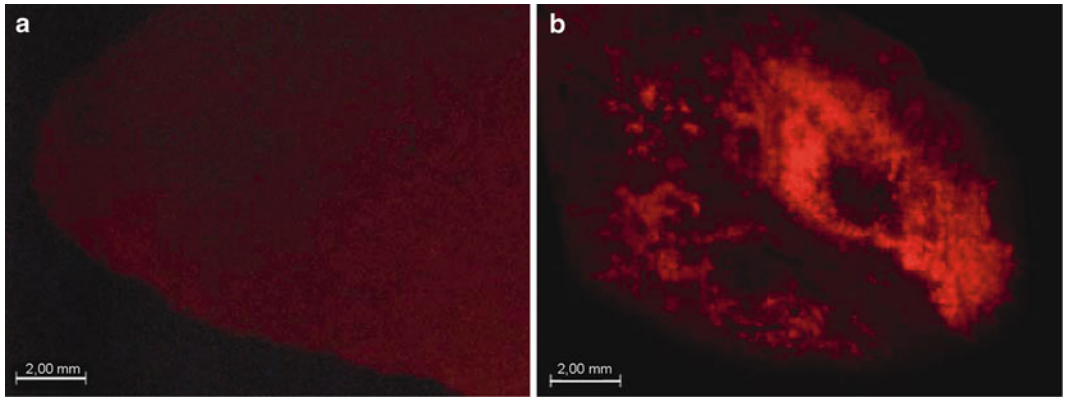

Fig. 2 Fluorescent micrographies of *Arabidopsis* leaves 3 days post-agroinfiltration. (**a**) Control leaf infiltrated with *Agrobacterium* without the plasmid containing the FP. (**b**) Leaf infiltrated with *Agrobacterium* with the plasmid containing the gene of interest fused to RFP (*red* fluorescent protein). Scale bar 2 mm

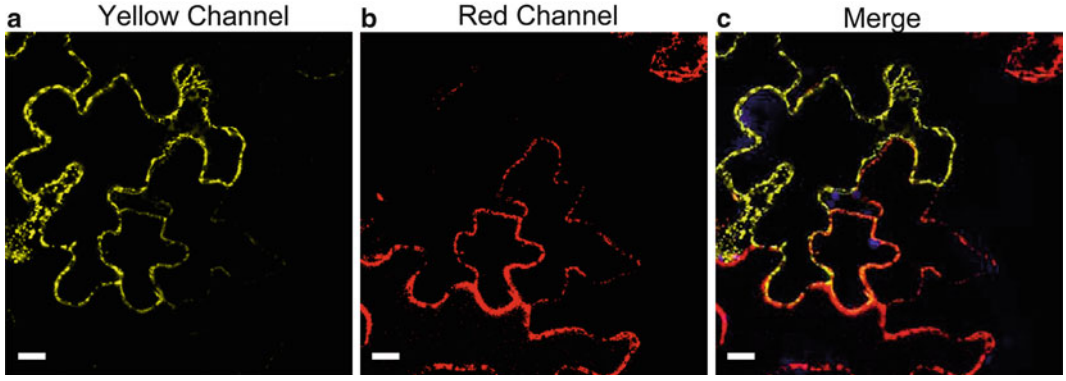

Fig. 3 Confocal scanning micrography of Arabidopsis leaves agroinfiltrated with ER-YFP and GI-RFP. (**a**) Yellow channel. (**b**) Red channel. (**c**) Merge channel. Scale bar 10 μm

4 Notes

1. *Arabidopsis* is a facultative long-day plant whose flowering is delayed in proportion to the light that the plant perceives. This photoperiod was chosen to promote leaf growth without altering drastically the flowering period. Arabidopsis plants are usually watered every 2 days.
2. Older plants with larger leaves also work, but the transformation efficiency decreases rapidly with the increase of plant age.
3. When Colony PCR is performed using Agrobacterium cells, the initial steps at 94 °C should be 10 min instead of 4 min, to promote the lysis of the cells. After this step, add the mix containing dNTPs, primers, and DNA taq polymerase.

4. *Agrobacterium tumefaciens* GV3101 is resistant to gentamicin (30 μg/mL) and rifampicin (10 μg/mL) and is sensitive to kanamycin so is a good strain for use with binary vectors that contains *npt II* gene.
5. Store several colonies for each vector, since there are differences in the expression levels of different colonies carrying the same binary vector.
6. The density of the bacterial suspension is also important for infiltration. Suspensions with an OD_{600nm} below 0.1 result in weak transgene expression. Infiltrations with bacterial suspensions with OD_{600nm} above 1.0 often result in tissue yellowing or wilting. The best results are obtained for suspension of OD_{600nm} between 0.4 and 0.6.
7. Agroinfiltration is preferably conducted during late afternoon or evening; therefore, T-DNA transfer occurs overnight.
8. Plants of similar size should be selected for optimal comparisons of experimental controls and tests. In addition, infiltration should be performed with leaves of the same age. Usually, leaves 6–8 are chosen for infiltration.
9. Observation can be performed using the whole plant without cutting the leaf, what allow to make a temporal analyzes.
10. The perfluorodecalin has a low surface tension [18]; therefore, it penetrates leaf stomatal pores and fills the intercellular air spaces of the mesophyll. Treatment with perfluorodecalin increases sensitive and improves the quality of the pictures.
11. The fluorescence is detected only in cells of the epidermis of the leaf. No fluorescence is found in leaf mesophyll cells, indicating that *Agrobacterium* was only able to transfer the DNA-T to cells of the leaf outer layers.
12. Simultaneous detection of RFP/mCherry and YFP or GFP is performed by combining the settings indicated above in the sequential scanning as instructed by the manufacturer.
13. When working with fusion proteins, the size of the protein of interest (Pi) fused to FP reporter should be analyzed by Western blot, to be sure that Pi was not separate of FP by proteolytic cleavage.

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